0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(7):2912–2921

doi: 10.1210/en.2002-0147

Nitric Oxide Inhibits Prolactin Secretion in Pituitary Cells Downstream of Voltage-Gated Calcium Influx

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The coupling between nitric oxide (NO)-cGMP signaling pathway and prolactin (PRL) release in pituitary lactotrophs has been established previously. However, the messenger that mediates the action of this signaling pathway on hormone secretion and the secretory mechanism affected, calcium dependent or independent, have not been identified. In cultured pituitary cells, basal PRL release was controlled by spontaneous voltage-gated calcium influx and was further enhanced by depolarization of cells and stimulation with TRH. Inhibition of constitutively expressed neuronal NO synthase decreased NO and cGMP levels and increased basal PRL release. The addition of a slowly releasable NO donor increased cGMP levels and inhibited basal PRL release in a time-dependent manner. Expression of inducible NO synthase also increased NO and cGMP levels and inhibited basal, depolarization-induced, and TRH-induced PRL release, whereas inhibition of this enzyme decreased NO and cGMP production and recovered PRL release. None of these treatments affected spontaneous and stimulated voltage-gated calcium influx. At basal NO levels, the addition of permeable cGMP analogs did not inhibit PRL secretion. At elevated NO levels, inhibition of cGMP production and facilitation of its degradation did not reverse inhibited PRL secretion. These experiments indicate that NO inhibits calcium-dependent PRL secretion in a cGMP-independent manner and downstream of voltage-gated calcium influx. (*Endocrinology* 144: 2912–2921, 2003)

THE ROLE OF nitric oxide (NO)/cGMP signaling pathway in control of secretory process has been studied in many neural and nonneural cell types, but the results are controversial. A number of experiments have suggested that this pathway stimulates secretion (1–6). Yet, in other experiments the addition of exogenous NO donors inhibited secretion (7–10). The finding that NO/cGMP signaling pathway could stimulate but also inhibit electrical activity and the associated Ca^{2+} influx (2, 11–15) could provide a rationale for such opposite actions of this pathway on secretion. However, in some cells NO donors reduce and inhibitors of NO synthase (NOS) enhance exocytosis in a Ca^{2+}-independent manner (1, 7, 9). Furthermore, it is debatable what signaling molecule, NO (11, 13, 14, 16) or cGMP (2, 7–9), mediates the action of this pathway on exocytosis.

Several factors could contribute to the complexity of NO/cGMP actions in target cells. The major mechanism of NO action is the activation of soluble guanylyl cyclase (sGC), leading to increase in cGMP production and activation of protein kinase G (PKG) (17). However, NO also exhibits sGC-independent intracellular actions in target cells, such as protein fatty acylation and protein *S*-nitrosylation (16, 18, 19). In addition, reactive nitrogen species can exert direct action on ion channels (11–14), and the cell type-specific expression of channels can determine the nature of NO/cGMP pathway (12, 15, 20, 21). Experimental factors could also account for such diverse actions of NO/cGMP signaling pathway on exocytosis. In experiments with exogenous NO donors, this could occur because of the rapid diffusion of NO across cell membranes to secondary targets that are difficult to identify. This is especially relevant for primary cultures containing several subpopulations of cells, where the paracrine interactions among cells further contribute to the complexity of NO/cGMP effects. Moreover, the levels and the kinetics of NO release reached by exogenous donors differ from NOS-derived NO. A large number of effector molecules for NO, cGMP, and PKG also favors the hypothesis of cell type-specific coupling between NO/cGMP signaling pathway and exocytosis. All together, these contradictions clearly indicate a need for multivariable approach in studying the NO/cGMP signaling pathway in a particular cell type.

Here we studied the role of NOS-sGC signaling pathway in control of exocytosis using rat pituitary lactotrophs as a cell model. These cells constitutively express neuronal NOS (nNOS) and spontaneous voltage-gated calcium influx controls the activity of this enzyme (22). Basal (in the absence of agonists) prolactin (PRL) secretion is also dependent on spontaneous electrical activity (23). Pituitary cells also express inducible NOS (iNOS) in response to stimulation with lipopolysaccharide and interferon-γ (LPS + IFN) (22, 24–26), which leads to inhibition of PRL release (26). Increase in NO levels by the addition of NO donors also inhibits PRL release (27). However, it is not clear what signaling molecules, NO and/or cGMP-PKG, accounts for this action in lactotrophs (28, 29). In addition, the dependence of NO/cGMP-induced inhibition of PRL secretion on calcium signaling has not been clarified.

Abbreviations: AG, Aminoguanidine; BK, big (maxi) Ca^{2+}-controlled K^+; 8-Br-cGMP, 8-bromo-cGMP sodium salt; DEA, *N*-ethylethanamine: 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; DPTA, 3,3′(hydroxynitrosohydrazino)bis-1-propan amine; IFN, interferon; iNOS, inducible NOS; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; ODQ, 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one; PKG, protein kinase G; PRL, prolactin; sGC, soluble guanylyl cyclase; Sp-8-Br-cGMPS, Sp-8-bromo-cGMP sodium salt.

To address these two important questions on the coupling of NOS-sGC signaling pathway and secretion, in our study with pituitary cells NO and cGMP levels were manipulated by stimulating iNOS expression, by inhibiting nNOS and iNOS activity, and by adding NO donors. Whenever possible, both messengers, NO and cGMP, and PRL secretion were measured from the same samples. To dissociate between NO and cGMP as potential messengers controlling the basal PRL release, intracellular cGMP levels were increased or decreased keeping the NO levels constant. The dependence of PRL secretion on NO/cGMP levels was examined in cells with basal intracellular calcium concentration ($[Ca^{2+}]_i$) and in cells with elevated $[Ca^{2+}]_i$ induced by the depolarization of cells and activation of TRH calcium-mobilizing receptors. In all experimental conditions, we observed no changes in the pattern of calcium signaling and a consistent inverse relationship between PRL and NO levels, but not between PRL and cGMP levels.

Materials and Methods

Cell cultures and treatments

Experiments were performed on anterior pituitary cells from normal postpubertal female Sprague Dawley rats obtained from Taconic Farms (Germantown, NY). Pituitary cells were dispersed as described previously (30) and cultured as mixed cells or enriched lactotrophs in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, and penicillin (100 U/ml) and streptomycin (100 μg/ml). A two-stage Percoll discontinuous density gradient procedure (30) was used to obtain enriched lactotrophs and further identification of lactotrophs in single cell calcium and NO studies was done by the addition of TRH (Peninsula Laboratories, Inc., Belmont, CA) at the end of recording. To express iNOS, cells were treated for 16 h with 30 μg/well LPS + 1000 IU/well IFN-γ (LPS + IFN) (Sigma, St. Louis, MO) (25). To elevate NO levels, cells were treated with 3,3′(hydroxynitroso-hydrazino)bis-1-propan amine (DPTA) and *N*-ethylethanamine:1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA), both from Alexis Biochemicals, San Diego, CA), an NO donor. NOS activity was inhibited by aminoguanidine (AG) (RBI/Sigma, Natick, MA) and N^w-nitro-L-arginine methyl ester (L-NAME, RBI/Sigma). Soluble guanylyl cyclase was inhibited by 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) from Calbiochem (San Diego, CA). cGMP levels were elevated by the addition of Sp-8-bromo-cGMP sodium salt (Sp-8-Br-cGMPS) and 8-bromo-cGMP sodium salt (8-Br-cGMP), both from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). The endogenous phosphodiesterase activity was not inhibited, unless otherwise indicated.

PRL, cGMP, cAMP, and nitrite measurements

Cells (1 million per well) were plated in 24-well plates in serum-containing M199 and incubated overnight at 37 C under 5% CO_2-air and saturated humidity. Before experiments, medium was removed and cells were washed and stimulated at 37 C under 5% CO_2-air and saturated humidity for 120 min if not otherwise stated. PRL and cyclic nucleotides were measured in incubation medium (released) and in cell content. Hormone secretion was also monitored using cell column perifusion experiments, as previously described (31). Briefly, 1.2×10^7 cells were incubated with preswollen cytodex-1 beads in 60-mm Petri dishes for 24 h. The beads were then transferred to 0.5 ml chambers and perifused with Hanks' M199 containing 25 mM HEPES, 0.1% BSA, and penicillin (100 U/ml)/streptomycin (100 μg/ml) for 2 h at a flow rate of 0.8 ml/min and at 37 C to establish stable basal secretion. Fractions were collected in 1-min intervals, stored at −20 C, and later assayed for PRL and cGMP contents using RIA. Primary antibody and standard for PRL assay were provided by the National Pituitary Agency and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA), ^{125}I-PRL was purchased from Perkin-Elmer Life Sciences (Boston, MA) and secondary antibody from Sigma. Cyclic nucleotides were determined using specific antisera provided by Albert Baukal (NICHD, Bethesda, MD). For measurements of NO production, sample aliquots were mixed with an equal volume of Greiss reagent containing 0.5% sulfanilamide and 0.05% naphthylethylenediamine in 2.5% phosphoric acid (all from Sigma), and the mixture was incubated at room temperature for 10 min and the absorbance measured at 546 nm. Nitrite concentrations were determined using a standard curve derived from increasing concentrations of sodium nitrite.

Single cell intracellular calcium and NO measurements

For $[Ca^{2+}]_i$ measurements, cells were incubated in Hanks' M199, supplemented with 2 μM fura-2 AM (Molecular Probes, Inc., Eugene, OR) at 37 C for 60 min. Coverslips with cells were then washed with Krebs-Ringer buffer and mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). Cells were examined under a ×40 oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 520 nm was measured. The ratio of light intensities, F_{340}/F_{380}, which reflects changes in Ca^{2+} concentration, was followed in several single cells simultaneously at the rate of one point per second. To measure NO production in single cells, the cells were loaded with 5 μM 4,5-diaminofluorescein diacetate by the same procedure as for fura-2. The excitation wavelength was 488 nm, and the emission was measured at 520 nm. The illumination and consequent fluorescence measurement occurred every 5 sec to minimize the effects of the light on the probe (32). Cells were stimulated with 10 μM DPTA or medium (controls) under identical conditions and the control curves were subtracted from those obtained upon NO donor stimulation.

Electrophysiological recordings

Calcium currents were measured using regular whole-cell perforated-patch recording technique as previously described (33). All voltage-clamp recordings were performed at room temperature using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, Foster City, CA) and were low-pass filtered at 2 kHz. A series resistance of less than 20 MΩ was reached 10 min following the formation of a gigaohm seal (seal resistance > 5 GΩ). When necessary, series resistance compensation was optimized and all current recordings were corrected for linear leakage and capacitance using a pulse/-4 procedure. An average membrane capacitance of 5.9 ± 0.2 pF was recorded in lactotrophs. Pulse generation and data acquisition were done with a PC equipped with a Digidata 1200 A/D interface in conjunction with Clampex 8 (Axon Instruments). The extracellular medium contained Krebs-Ringer's solution with 20 mM tetraethylammonium, 2 mM $CaCl_2$, and 1 μM tetrodotoxin (pH adjusted to 7.4 with NaOH) and the pipette contained (in mM): 120 CsCl, 20 tetraethylammonium-Cl, 4 $MgCl_2$, 10 EGTA, 9 glucose, 20 HEPES, 0.3 Tris-GTP, 4 Mg-ATP, 14 $CrPO_4$, and 50 U/ml creatine phosphokinase (pH adjusted to 7.2 with Tris base).

Calculations

Calcium, current, and single cell NO data are shown as representative traces or computer derived means. cGMP data are shown as total (cell content + medium) nucleotide levels, PRL data are shown separately for incubation/perifusion medium (released hormone) and cell content, and NO data are shown exclusively from measurements in medium. Unless otherwise indicated, the results shown are means ± SE from at least three experiments, each done in sextuplicate incubation. Significant differences, with $P < 0.05$, were determined by one-way ANOVA with Neuman-Keuls multiple comparison test. Linear and log-linear regression analyses were used to correlate variables, and the strength of correlation was expressed as Pearson's R coefficient (KaleidaGraph, Reading, PA).

Results

Role of calcium influx on basal and TRH-induced $[Ca^{2+}]_i$ and PRL release

Pituitary lactotrophs bathed in calcium-containing medium exhibit spontaneous firing of bursting type of action

potentials accompanied with $[Ca^{2+}]_i$ transients (23). Figure 1, A–C, *left traces*, illustrates typical patterns of voltage-gated $[Ca^{2+}]_i$ transients in single lactotrophs. The addition of TRH increased $[Ca^{2+}]_i$ above the basal level in a biphasic manner (an early spike accompanied with a plateau; Fig. 1A, *left trace*). TRH also induced biphasic changes in PRL release in perifused pituitary cells (Fig. 1A, *right panel*). The addition of 2 mM EGTA reduced free extracellular calcium concentration to about 100 nM, abolished spontaneous $[Ca^{2+}]_i$ transients (Fig. 1B, *left trace*), and dramatically reduced PRL release. As shown in Fig. 1B, *right panel*, the residual basal PRL release

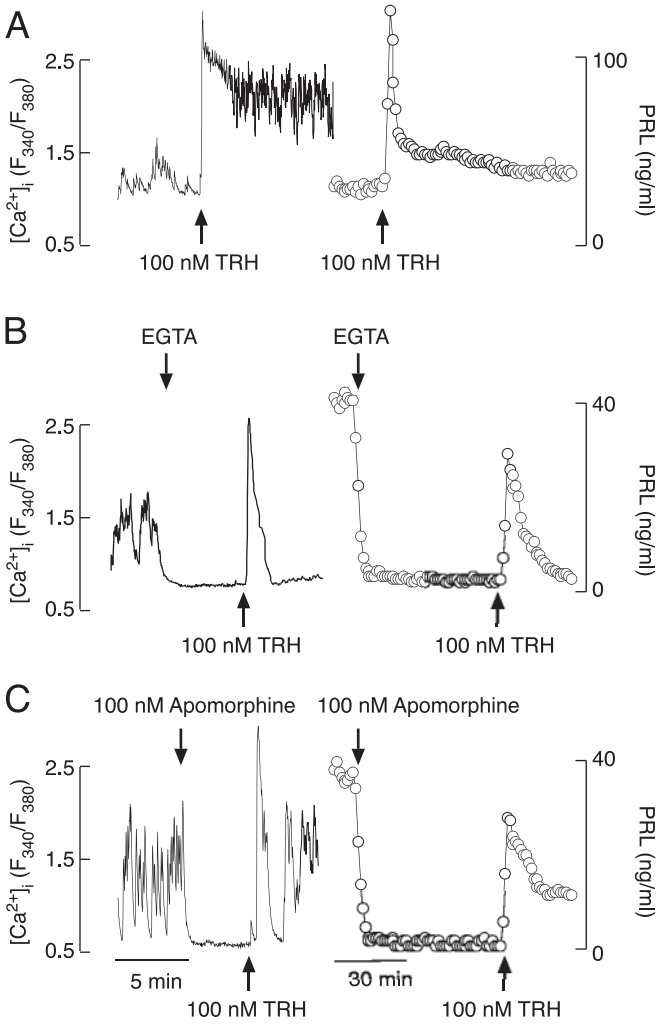

FIG. 1. Dependence of basal and TRH-induced PRL release on extracellular calcium. A, Biphasic (an early spike followed by a plateau) effect of TRH on $[Ca^{2+}]_i$ (*left panel*) in single lactotrophs and PRL release (*right panel*) in perifused pituitary cells bathed in 1.8 mM calcium-containing medium. B, Effects of 2 mM EGTA on spontaneous $[Ca^{2+}]_i$ transients (*left panel*) and basal PRL release (*right panel*) in lactotrophs bathed in 1.8 mM Ca^{2+}-containing medium. Notice that in the presence of EGTA, TRH-induced spike in $[Ca^{2+}]_i$ and PRL release is preserved, but the sustained calcium and secretory responses were abolished. C, Inhibition of spontaneous $[Ca^{2+}]_i$ transients (*left panel*) and basal PRL release (*right panel*) by apomorphine, a specific D_2 receptor agonist. The presence of apomorphine did not affect TRH-induced spike in $[Ca^{2+}]_i$ and PRL response, whereas the plateau responses were partially recovered. Traces shown are representative from at least 25 cells per treatment (calcium recordings) and three independent experiments (secretion).

was less than 3% of that observed in cells perifused in calcium-containing medium. The addition of TRH in the presence of EGTA induced a monophasic rise in $[Ca^{2+}]_i$ and PRL release, *i.e.* the sustained responses were abolished, indicating that the spike phase in both calcium signaling and secretion is controlled by calcium mobilization from intracellular pools and the plateau phase is dependent on calcium influx. Activation of dopamine receptors by apomorphine also inhibited spontaneous $[Ca^{2+}]_i$ transients and almost completely abolished basal PRL release (Fig. 1C). In the presence of apomorphine, TRH-induced spike $[Ca^{2+}]_i$ and PRL responses were accompanied with a partial recovery of sustained calcium influx and PRL release. These results are consistent with literature (reviewed in Ref. 34), confirming that basal PRL secretion is controlled by calcium influx and that TRH acts as a calcium-mobilizing agonist during the initial phase of stimulation but also further promotes calcium influx.

Effects of NO donors on PRL secretion

The addition of 10 μM DPTA, a slow releasable NO donor, significantly increased nitrite levels in culture medium (Fig. 2A) and cGMP production (Fig. 2B), both in a time-dependent manner. This was accompanied with a significant decrease in PRL release (Fig. 2C), whereas the cellular PRL content was not affected (Fig. 2D), indicating that NO and/or cGMP inhibit the exocytotic pathway but not *de novo* PRL package/synthesis. The rate of DPTA-dependent NO release decreased dramatically after 0.5 h (Fig. 2A), coinciding with the onset of inhibition of PRL release (Fig. 2C).

The DPTA-induced NO increase was also detectable in the

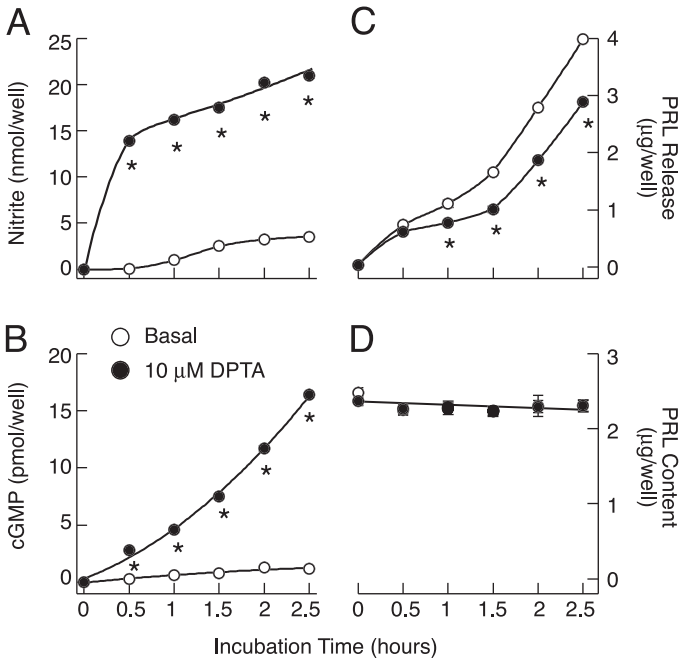

FIG. 2. Inhibition of PRL release in pituitary cells in static cultures by DPTA, a slowly releasable NO donor. A–C, Time-course of nitrite (A) and cGMP (B) accumulation, and PRL release (C) in controls and cells stimulated with 10 μM DPTA. D, The lack of effects of DPTA on PRL cell content. Unless otherwise indicated, in this and following figures *asterisk*s indicate significant differences among pairs.

cytosol of single lactotrophs (Fig. 3A). However, DPTA did not affect the pattern of spontaneous $[Ca^{2+}]_i$ transients. Figure 3B illustrates the lack of effects of 10 μM DPTA on $[Ca^{2+}]_i$ signaling. When exposed to 50 μM DPTA, the pattern of calcium transients was affected in none of 54 analyzed cells, and when treated with 100 μM DPTA, 35 out of 43 cells did not change the pattern of signaling, whereas the residual cells showed an increase or abolishing of spiking. Similarly, the addition of 50 μM DEA, a rapidly releasable NO donor, was ineffective in 90 out of 98 cells.

In the presence of DPTA, apomorphine inhibited Ca^{2+} influx, and TRH stimulated Ca^{2+} mobilization, both in a manner similar to that observed in control cells (Fig. 3B *vs.* Fig. 1). In parallel to calcium recordings, electrophysiological measurements indicated that DPTA did not change the pattern of voltage-gated calcium current (Fig. 3C) and the I-V characteristics of this current (D), whereas apomorphine inhibited calcium current. These results indicate that, in contrast to dopamine-2 receptors, NO/cGMP signaling pathway attenuates Ca^{2+}-dependent PRL release downstream of voltage-gated Ca^{2+} influx.

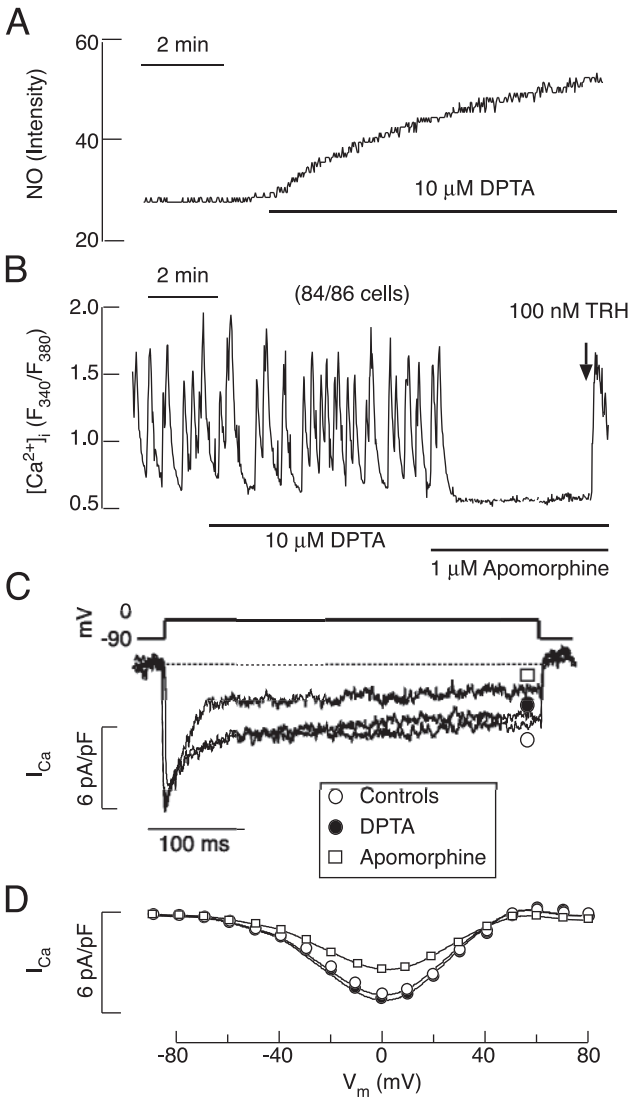

FIG. 3. Effects of DPTA on intracellular NO and voltage-gated calcium signaling in single lactotrophs. A, DPTA-induced rise in cytosolic NO, measured in cells loaded with 4,5-diaminofluorescein diacetate (DAF-2 DA). B, The lack of effect of 10 μM DPTA on spontaneous $[Ca^{2+}]_i$ transients. Notice that in the same cell, apomorphine was able to inhibit calcium transients in the presence of DPTA. Traces shown are representative and numbers above traces indicate the fraction of cells in which the pattern of Ca^{2+} signaling was not changed. C, Representative voltage-gated I_{Ca} traces in a lactotroph before and during DPTA (10 μM) or apomorphine (100 nM) stimulation. D, Current-voltage relation of the sustained voltage-gated I_{Ca}. Traces shown are representative from 47 cells (A), 86 cells (B), and 9 cells (C).

Dependence of basal PRL release on nNOS activity

Pituitary lactotrophs constitutively express nNOS (22). To attenuate the activity of this enzyme, cells were treated with L-NAME, a nonspecific NOS inhibitor. As shown in Fig. 4A, the addition of L-NAME to the bath medium decreased NO/cGMP levels and increased PRL release, both in a concentration-dependent manner and with IC_{50}s typical for this compound (35). As in experiments with DPTA, the treatment of cells with L-NAME did not change the pattern of spontaneous $[Ca^{2+}]_i$ transients (Fig. 4B, *upper trace*). In quiescent lactotrophs (about 20–30% of cells), L-NAME did not initiate Ca^{2+} influx (not shown). Furthermore, L-NAME was ineffective in changing the pattern of Bay K 8644 (an L-type calcium channel agonist) induced $[Ca^{2+}]_i$ transients (Fig. 4B, *bottom trace*). The effects of 5 mM L-NAME on PRL release were not observed when the NO levels were elevated with 10 μM DPTA (DPTA alone = 2.19 ± 0.09, DPTA + L-NAME = 2.21 ± 0.13 μg/well), arguing against the hypothesis that L-NAME exhibits nonspecific actions on exocytosis at that concentration. These results indicate that basal nNOS activity contributes to the control of PRL secretion in cultured pituitary cells.

Dependence of PRL release on iNOS expression

Pituitary cells express iNOS when stimulated with LPS + IFN (22, 25, 26). As shown in Fig. 5, this resulted in a significant increase in NO (A) and cGMP production (B) and a significant decrease in basal PRL release (C), whereas cellular PRL content was not affected (D). Down-regulation of iNOS activity by 1 mM AG, a relatively specific iNOS inhibitor (36), significantly decreased the NO (Fig. 5A) and cGMP production (B). This led to the recovery in PRL release (Fig. 5C) without affecting PRL content (D). Similar effects were observed in cells treated with L-NAME (not shown). Changes in the levels of endogenously released NO by expressing iNOS did not affect the percentage of lactotrophs exhibiting spontaneous $[Ca^{2+}]_i$ transients (128 out of 163 cells in control cultures *vs.* 54 out of 71 cells in iNOS-expressing cells), nor the pattern of $[Ca^{2+}]_i$ transients in iNOS-expressing cells treated with 5 mM L-NAME (Fig. 6A) or 1 mM AG (not shown).

To compare more directly the relationship between $[Ca^{2+}]_i$ signaling and secretion in iNOS-expressing cells, the mean $[Ca^{2+}]_i$ response was generated from a pool of lactotrophs in controls and iNOS-expressing cells, and the rate of PRL secretion was measured in perifused pituitary cells. Figure 6B shows that the mean basal $[Ca^{2+}]_i$ levels were comparable in both cultures (*left panel*), whereas PRL release (*right panel*)

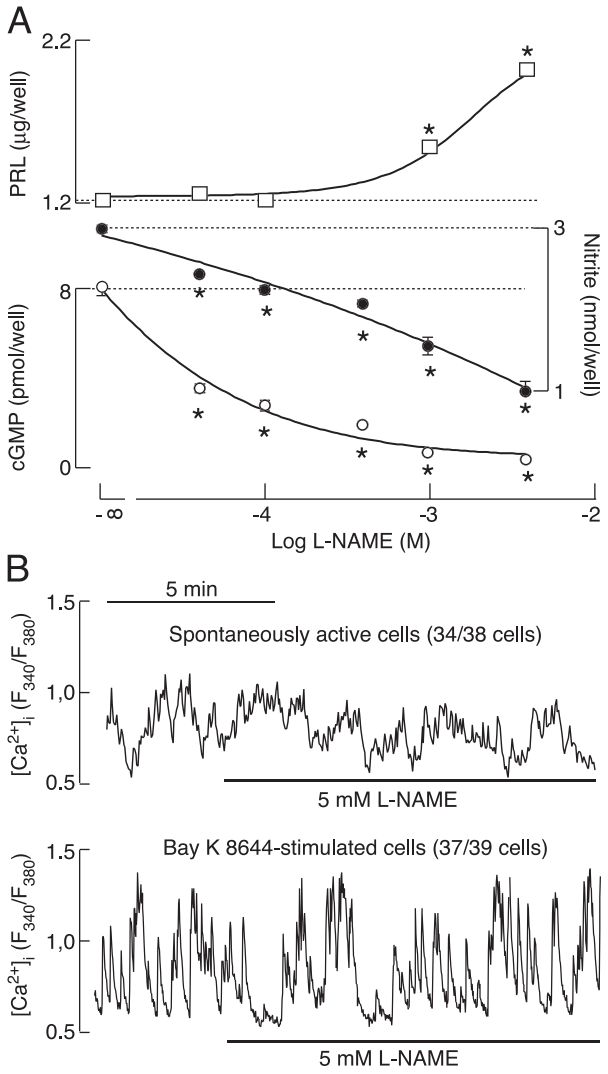

FIG. 4. Dependence of PRL release on basal NO production in pituitary cells. A, Concentration-dependent effects of L-NAME, an NOS inhibitor, on basal NO (*central curve*), cGMP (*bottom curve*), and PRL release (*upper curve*). In this and following figures, cells in static cultures were incubated for 2 h in the presence of L-NAME and PRL, nitrite, and cGMP levels were determined from the same samples, unless otherwise indicated. B, The lack of effects of L-NAME on $[Ca^{2+}]_i$ signaling in spontaneously active (*upper trace*) and Bay K 8644-stimulated (*lower trace*) lactotrophs. Traces shown are representative and numbers above traces indicate the number of cells in which the pattern of Ca^{2+} signaling was not changed.

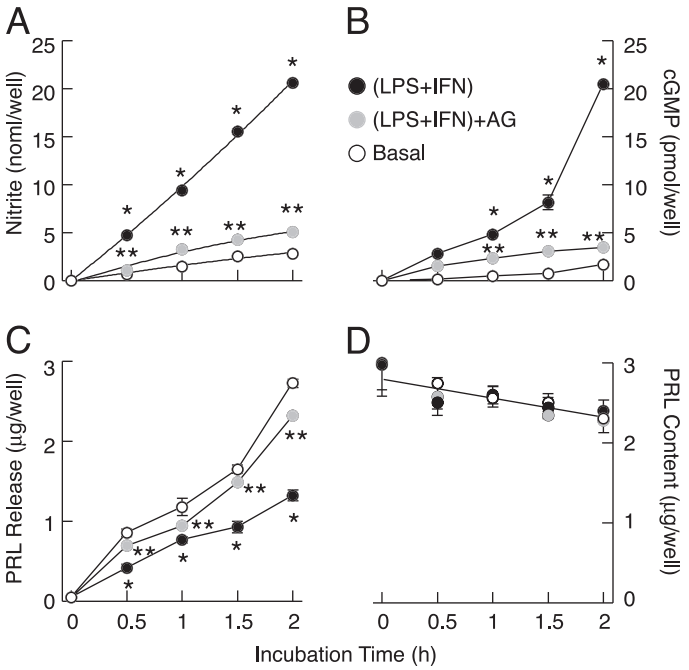

FIG. 5. Time-course of NO (A), cGMP accumulation (B), and PRL release (C), and PRL content (D) in controls and cells expressing iNOS. To express iNOS, cells in static cultures were treated with 30 μg/well LPS and 1000 IU/well IFN overnight. To inhibit iNOS, cells were treated with 1 mM AG. At 0 time point, cells were washed twice and incubated for indicated times. *, Significant differences between basal and (LPS + IFN)-treated cells; **, significant differences between (LPA + IFN)- and (LPS + IFN) + AG-treated cells.

was lower in iNOS-expressing cells. Lactotrophs from both cultures also responded to TRH with highly comparable $[Ca^{2+}]_i$ signaling profiles (Fig. 6C, *left panel*), whereas TRH-induced peak PRL release was inhibited in iNOS-expressing cells (*right panel*). As shown earlier (Fig. 1), TRH-induced peak $[Ca^{2+}]_i$ response predominantly reflects Ca^{2+} mobilization from intracellular pool, suggesting that NO/cGMP signaling pathway also inhibits receptor-controlled PRL release without affecting agonist-induced Ca^{2+} mobilization.

In further experiments, we depolarized controls and iNOS-expressing cells by high potassium and Bay K 8644 and compared $[Ca^{2+}]_i$ signals and secretory profiles. In all lactotrophs studied, 25 mM K^+ induced a nonoscillatory rise in $[Ca^{2+}]_i$ and the mean profiles of signals were highly comparable in both cultures (Fig. 7A, *left panel*). In contrast, K^+-induced PRL secretion was dramatically reduced in iNOS-expressing cells (Fig. 7A, *right panel*). Bay K 8644 initiated spiking in quiescent cells and increased the frequency of $[Ca^{2+}]_i$ transients in spontaneously active cells in both cultures (not shown), with comparable mean values of $[Ca^{2+}]_i$ (Fig. 7B, *left panel*). As in K^+-depolarized cells, Bay K 8644-induced PRL secretion was reduced in iNOS-expressing cells (Fig. 7B, *right panel*).

NO inhibits PRL secretion in a cGMP-independent manner

To dissociate which of the two molecules, NO and/or cGMP, signals for inhibition of PRL release, three different experimental approaches were used. We initially analyzed the effects of elevation in cGMP levels on the patterns of calcium signaling in single cells and PRL secretion in perifused pituitary cells. Cells were treated with two permeable cGMP analogs, 8-Br-cGMP and Sp-8-Br-cGMPS. 8Br-cGMP did not change the pattern of spontaneous and Bay K-8644-induced Ca^{2+} transients (Fig. 8A). Basal PRL release was also not affected by 8Br-cGMP in 0.1–5 mM concentration range (Fig. 8, B and C). The other permeable cGMP analog, Sp-8-Br-cGMPS, was also ineffective (Fig. 8D).

Pharmacologically high NO levels are known to decrease rather than increase cGMP levels without affecting sGC activity, and such induced desensitization of the cGMP response is caused by the phosphorylation and subsequent increase in the activity of phosphodiesterase 5 (37). In ac-

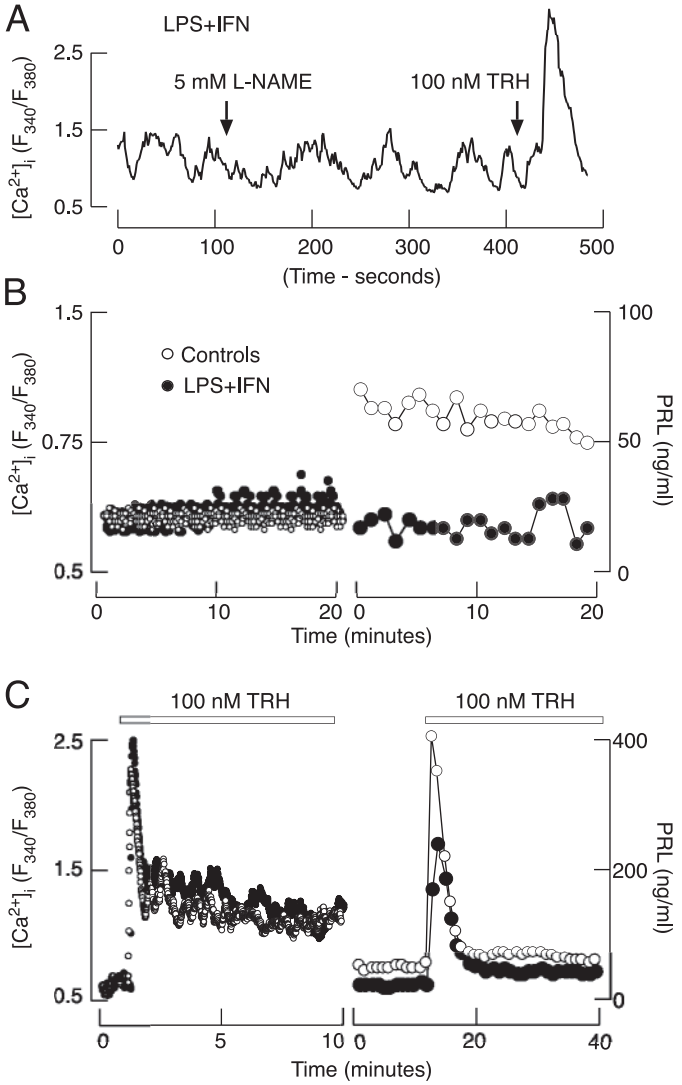

FIG. 6. Characterization of calcium signaling and secretion in iNOS-expressing pituitary cells. A, The lack of effects of L-NAME on the pattern of spontaneous $[Ca^{2+}]_i$ transients in single lactotrophs. The trace shown is representative from 43 out of 54 cells. In 11 cells, stimulation or inhibition of $[Ca^{2+}]_i$ transients was observed. B, *Left panel*, Basal $[Ca^{2+}]_i$ in controls and cells expressing iNOS. B, *Right panel*, Basal PRL release by perifused control and iNOS-expressing pituitary cells. C, *Left panel*, TRH-induced biphasic $[Ca^{2+}]_i$ response in controls and iNOS-expressing cells. C, *Right panel*, TRH-induced PRL release by perifused control and iNOS-expressing pituitary cells. In B and C, $[Ca^{2+}]_i$ values shown are means from at least 17 cells, and the PRL secretory profiles are representative from three experiments.

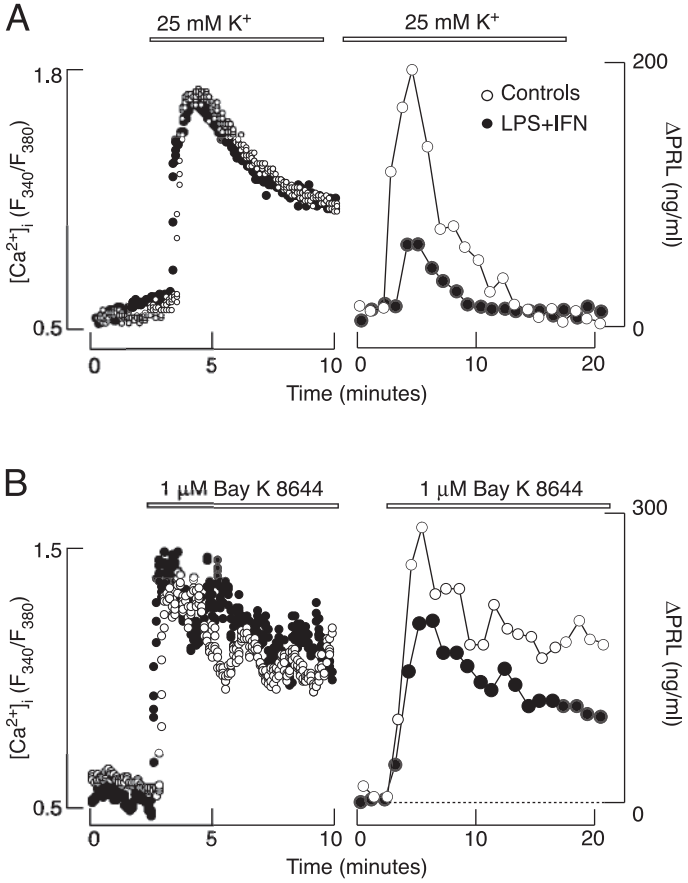

FIG. 7. Effects of depolarization on $[Ca^{2+}]_i$ and PRL release in controls and iNOS-expressing cells. A and B, *Left panels*, mean $[Ca^{2+}]_i$ values in controls and iNOS-expressing cells in response to stimulation with 25 mM K^+ (A, n = 26) and 1 μM Bay K 8644 (B, n = 27). A and B, *Right panels*, Rate of PRL release by perifused control and iNOS-expressing cells stimulated with 25 mM K^+ (A) and 1 μM Bay K 8644 (B). To exclude the impact of iNOS expression in unstimulated cells, basal PRL secretion was subtracted.

cordance with this, in pituitary cells with inhibited phosphodiesterase activity DPTA induced a sigmoidal dose-dependent increase in cGMP accumulation (not shown), whereas in cells bathed in medium without phosphodiesterase inhibitors, the bidirectional effects of NO on cGMP accumulation were observed (Fig. 9B). In both experimental conditions, stimulation of pituitary cells with increasing concentrations of DPTA led to a proportional increase in nitrite levels and progressive decrease in PRL release. Figure 9A illustrates effects of DPTA on NO levels (A) and PRL release (C), both in cells without inhibited phosphodiesterases. Regression analysis revealed a log-linear correlation between nitrite levels and PRL release in the concentration-range studied (R = 0.94) and no significant correlation between cGMP levels and PRL release (R = 0.59).

Because high NO-induced facilitation of cGMP degradation reduced but did not completely abolish the NO-stimulated cGMP levels, the residual cGMP levels still could contribute to the inhibition of PRL secretion. To clarify this issue, in final experiments we treated iNOS-expressing cells with ODQ, an inhibitor of sGC. As shown in Fig. 10A, ODQ inhibited cGMP production in a concentration-dependent manner and with an IC_{50} of about 300 nM. cGMP production in control cells was also reduced by ODQ treatment in a dose-dependent manner. At 100-μM concentrations of ODQ, no difference in cGMP production was observed between controls and iNOS-expressing cells. Such a dramatic inhibition of sGC in iNOS-expressing cells occurred at elevated NO levels, which were not affected by ODQ (Fig. 10B). In parallel to NO levels, PRL release was not changed by ODQ treatment in both experimental groups (Fig. 10C), indicating that ODQ does not have an effect on PRL secretion independent of the status of NO/cGMP signaling pathway. All together,

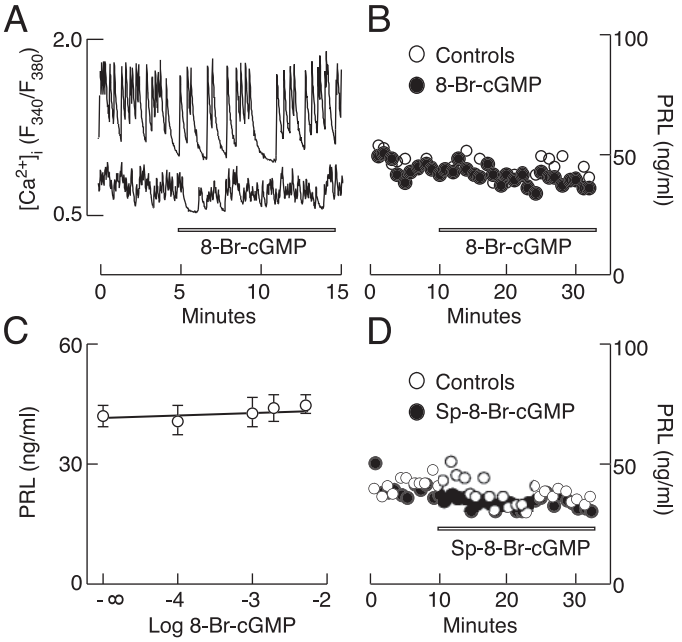

FIG. 8. Effects of elevated cGMP levels on calcium signaling and PRL release in normal pituitary cells. A, The lack of effects of 2 mM 8Br-cGMP on calcium signaling in spontaneously active (*bottom trace*; 55 out of 57 cells) and 1 μM Bay K 8644-stimulated cells (*upper trace*; 36 out of 37 cells). B and D, The lack of effects of 2 mM 8Br-cGMP (B) and 0.1 mM Sp-8-Br-cGMP (D) on PRL release in perifused pituitary cells. C, Dose-dependent effects of 8-Br-cGMP on PRL release in pituitary cells in static culture.

these results indicate that NO, rather than cGMP, mediates the action of this signaling pathway on PRL release.

Discussion

Here we confirmed previous findings by other laboratories that the pituitary NO/cGMP signaling pathway is involved in control of PRL secretion. We also present three novel findings. First, this signaling pathway does not affect spontaneous depolarization- and TRH-induced calcium influx, but it inhibits hormone release downstream of calcium signaling. Second, unlike the effects of dopamine-2 receptor agonist, this inhibition is partial and requires time to develop. Third, NO inhibits PRL secretion independently of cGMP signaling pathway. These observations are in agreement with hypothesis that NO is not a feedback controller of pacemaker activity in physiological conditions, but rather it might serve as a feedforward tonic controller of calcium influx-mediated secretion rendering the cells protected from an excessive PRL release. In immune and inflammatory reactions, iNOS-derived NO further inhibits PRL release.

The expression and operation of NO-sensitive sGC signaling system in anterior pituitary and immortalized pituitary cells is well established. Pituitary cells constitutively express Ca^{2+}-dependent nNOS and Ca^{2+}-independent iNOS in response to LPS + IFN stimulation (22, 24, 25, 38–43). The activity of these enzymes was confirmed by measurements of NO, NO_2, and NO_3 under different experimental paradigms (22, 44, 45). sGC is also expressed in pituitary tissue and dispersed cells, enriched lactotrophs and somatotrophs, and GH_3 immortalized cells, and this enzyme is exclusively responsible for cGMP production in unstimulated cells (22). Inhibition of voltage-gated calcium influx by nifedipine and by the addition of EGTA inhibits basal NOS-sGC activity (22). Basal growth hormone and PRL secretion is also controlled by spontaneous Ca^{2+} influx. Somatotrophs and lactotrophs fire the plateau-bursting type of action potentials associated with high amplitude Ca^{2+} transients and abolition of such firing leads to inhibition of basal hormone secretion (23, 46).

The parallelism in the actions of voltage-gated Ca^{2+} influx on nNOS activity and PRL secretion does not necessarily indicate that NO/cGMP signaling pathway influences Ca^{2+}-controlled exocytosis in lactotrophs. More conclusive evidence for such coupling was observed in experiments in hemipituitaries, where the addition of NO donors decreased and inhibition of endogenous NO production increased basal PRL secretion (27). The expression of iNOS in pituitary cells was also accompanied with inhibition of basal PRL secretion (26). Others, however, have observed that inhibition of endogenous NOS and the addition of IL-1β inhibits PRL secretion, whereas the addition of NO donors stimulates basal PRL release and enhances IL-1β-induced PRL release (47), suggesting that NO/cGMP signaling pathway facilitates Ca^{2+}-influx-dependent PRL secretion. We observed a consistent inhibitory effect of elevated NO/cGMP on PRL secretion in multivariable experimental conditions with NO and cGMP levels in physiological, pathophysiological (in iNOS-expressing cells), and pharmacological (by the addition of NO donors) conditions. We also show that NO/cGMP in all concentrations studied attenuates but does not abolish Ca^{2+} influx-dependent hormone secretion, in contrast to the activation of dopamine-2 receptors.

Calcium dependence of NO/cGMP action on PRL secretion has not been extensively studied. In immortalized pituitary cells, it has been suggested that cGMP facilitates voltage-gated Ca^{2+} influx (48), an action not consistent with inhibition of PRL release by NO/cGMP observed in native lactotrophs. Calcium measurements in mixed population of pituitary cells in suspension have indicated a small and transient decrease in basal $[Ca^{2+}]_i$ upon NO donor treatment (28), in contrast to sustained (for several hours) inhibition of PRL release. This observation suggests that in mixed population of anterior pituitary cells NO/cGMP may affect the pacemaker activity in cell types other than lactotrophs. Here we show for the first time in identified single lactotrophs that increase in NO/cGMP levels by expression of iNOS and by addition of NO donors, and inhibition of nNOS and iNOS activity by AG and L-NAME, did not change the pattern of voltage-gated calcium currents and the associated $[Ca^{2+}]_i$ transients, or the percentage of cells exhibiting spontaneous $[Ca^{2+}]_i$ transients. In the same cells, we were able to show changes in NO levels, strongly supporting the hypothesis that NO/cGMP pathway controls PRL secretion downstream of voltage-gated Ca^{2+} influx. This hypothesis was further confirmed in experiments with depolarization-induced Ca^{2+} influx and receptor-induced Ca^{2+} mobilization, which were comparable in controls and iNOS-expressing cells, whereas PRL release was suppressed in iNOS-expressing cells.

At the present time, it is not clear why elevated NO-cGMP

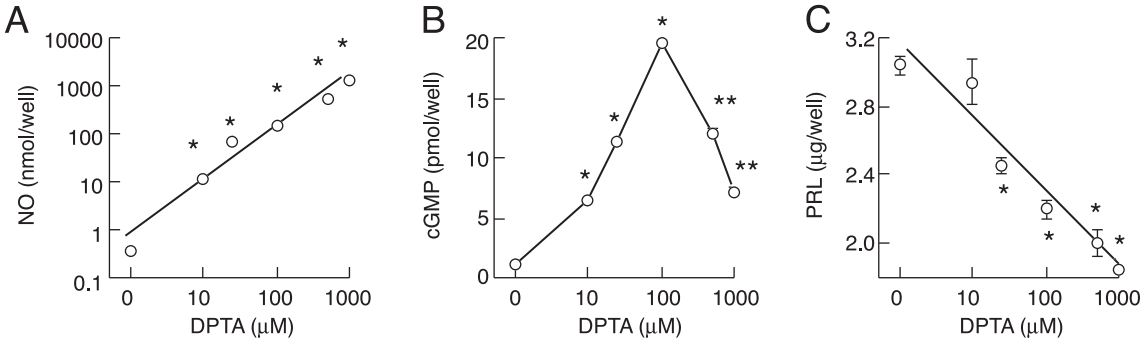

FIG. 9. Dissociation between NO and cGMP levels in pituitary cells stimulated with increasing concentrations of DPTA. A, Linear relationship between DPTA concentrations and NO levels in medium. B, Biphasic effects of DPTA on cGMP production. C, Inverse relationship between DPTA concentrations and PRL release. *, Significant differences between DPTA-treated and untreated cells; **, significant differences between 100 μM DPTA-treated cells *vs.* 500 μM and 1000 μM DPTA-treated cells.

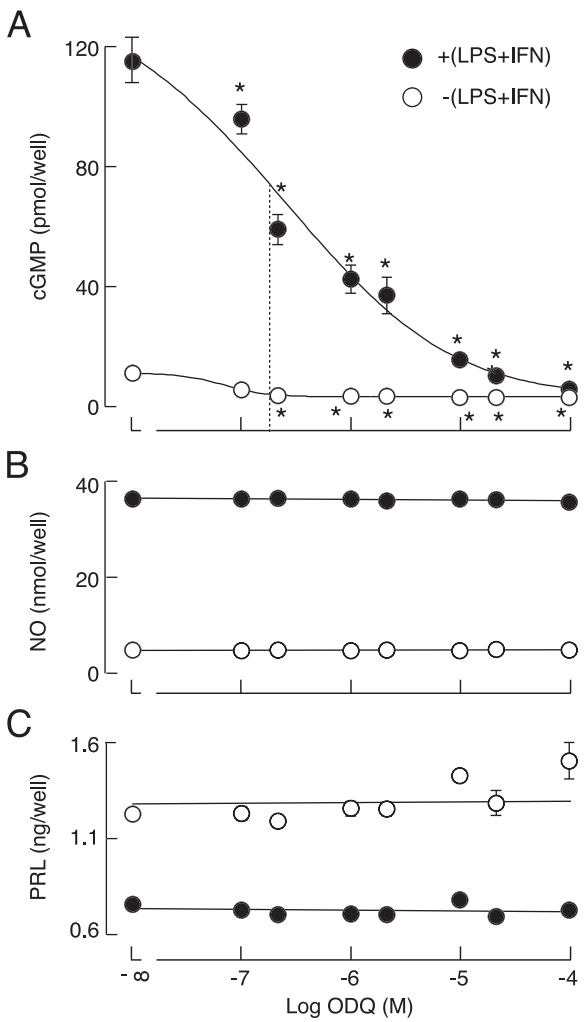

FIG. 10. Independence of PRL release on cGMP levels in pituitary cells expressing iNOS. Concentration dependence of ODQ, an sGC inhibitor, on cGMP levels (A), NO levels (B), and PRL release (C) in controls [-(LPS + IFN)] and iNOS-expressing cells [+ (LPS + IFN)]. The *dashed vertical line* denotes the calculated EC_{50} for ODQ effects on cGMP production in iNOS-expressing cells.

levels do not affect the pacemaker activity and the associated Ca^{2+} signaling in lactotrophs as observed in some other cell types. For example, in cells expressing cyclic nucleotide-gated channels, cGMP facilitates pacemaking and calcium influx directly (20), whereas in cells expressing big (maxi) Ca^{2+}-controlled K^+ (BK) channels PKG is mediating the inhibitory action of cGMP on electrical activity and Ca^{2+} signaling (12, 15, 21). Elegant experiments with vascular smooth muscle cells have also demonstrated the relevance of PKG I isoform in relaxation of cells (21), consistent with the role of this enzyme in regulation of BK channels (15). Lactotrophs also express cyclic nucleotide-like channels (49) and BK channels (33), but we were unable to observe a consistent activation of these channels by 8-Br-cGMP and NO donors (Van Goor, F., M. Tomić, and S. S. Stojilkovic, unpublished observations).

Three lines of evidence shown here indicate that NO rather than cGMP inhibits PRL secretion downstream of voltage-gated Ca^{2+} influx. First, in our experiments with fast delivery of compounds during perifusion, as well as in dispersed pituitary cells in static cultures, two permeable cGMP analogs were unable to alter PRL secretion and NO levels when added in 0.1–5 mM concentration-range. An earlier study has shown that in intact pituitary tissue the exogenous monobutyril cGMP in high concentrations significantly inhibited PRL secretion, whereas in lower concentration this compound was ineffective (27). Second, as in other cell types (37), in pituitary cells expressing iNOS and bathed in medium without phosphodiesterase inhibitors, high NO levels decreased cGMP levels. Such partial inhibition of cGMP production was not associated with recovery of PRL release, which was inversely correlated with NO levels. Third, using ODQ, an sGC inhibitor, we were able to inhibit the enzyme activity in a concentration-dependent manner, but this inhibition did not change the pattern of NO and PRL release.

Further studies are required to clarify how NO exerts its action on PRL secretion, directly on the exocytotic system or through other effector molecules. In accordance with the first hypothesis, NO was found to modulate a late step in exocytosis in chromaffin cells, probably by regulation of the emptying of secretory vesicles (9). Consistent with the second hypothesis, NO exhibits several sGC-independent actions, such as posttranslational modification of proteins [ADP ribosylation (50), fatty acylation (18), and *S*-nitrosylation (16, 19)]. The reactive nitrogen species can also alter the levels of free radicals, which could mediate the action of NO on secretion and other cellular processes. For example, the

posttranslational modification of sulfhydryl groups by NO was suggested to alter synaptic protein interactions that regulate synaptic transmission (16). In our experimental conditions, the effects of DPTA on PRL release were much slower compared with NO release. Also, cells expressing iNOS were under prolonged and tonic exposure to elevated NO levels. Both observations are consistent with the hypothesis of indirect effects of NO on exocytosis.

Activation of NO production by spontaneous voltage-gated Ca^{2+} influx and the subsequent control of PRL secretion by NO without affecting the pattern of Ca^{2+} influx suggest a feedforward rather than a feedback controller function of this messenger in lactotrophs. In this scenario, shown in Fig. 11, spontaneous pacemaker activity in lactotrophs stimulates secretion through voltage-gated Ca^{2+} influx, and at the same time stimulates nNOS activity and accounts for other Ca^{2+}-controlled cellular functions. Unlike dopamine, increase in NO production does not block PRL secretion, but attenuates it. In accordance with these observations, it has been published recently that NO modifies a late step in Ca^{2+}-controlled exocytosis by decreasing a quantum of hormone release, probably by influencing the fusion pore open time (9). The expression of iNOS further inhibits Ca^{2+}-controlled PRL release during immune and inflammatory reactions. This action is disconnected from voltage-gated Ca^{2+} influx, because iNOS does not require a rise in $[Ca^{2+}]_i$ for its activation and does not modify Ca^{2+} influx. The lack of the effects of NO/cGMP on pacemaker activity provides the opportunity for lactotrophs to keep operative other voltage-gated Ca^{2+} influx-controlled functions, including gene expression and cGMP-PKG-controlled cellular processes. The feedforward controller function is consistent with protection of cells from an excessive PRL release, whereas the hypothalamic (by dopamine and somatostatin) and pituitary (by endothelins) inhibition of PRL release is a switch-off type of control, because it abolishes all voltage-gated Ca^{2+} influx-controlled cellular functions by blocking the pacemaking activity. We may speculate that the NO/cGMP signaling pathway does not substitute for hypothalamic control of PRL secretion, but provides an additional mechanism for controlling hormone secretion at pituitary level without altering spontaneous electrical activity.

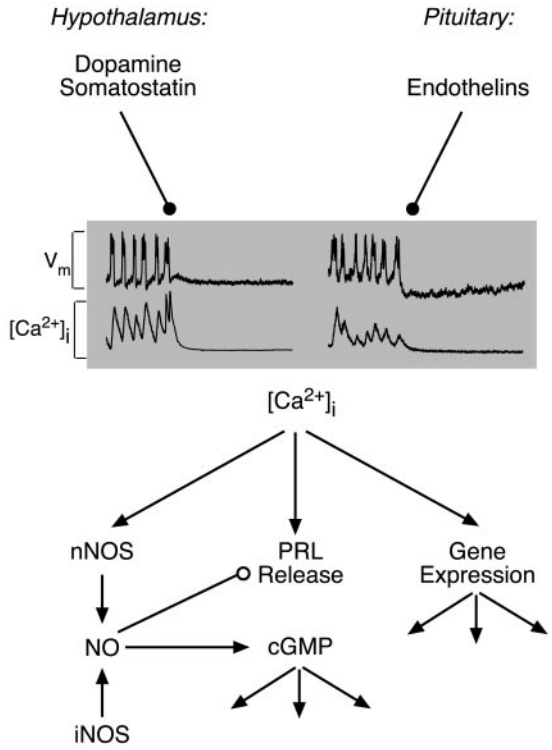

FIG. 11. Spontaneous voltage-gated Ca^{2+} influx and Ca^{2+}-controlled cellular functions in pituitary lactotrophs. Traces shown in gray area are obtained in simultaneous measurements of membrane potential (V_m) and $[Ca^{2+}]_i$ from single lactotrophs. Such spontaneous activity in lactotrophs is sufficient to control several cellular functions, including the activity of nNOS, PRL secretion, and gene expression/protein synthesis. The nNOS-derived NO stimulates sGC, but also down-regulates PRL release. The direct action of NO on PRL release downstream of Ca^{2+} influx is consistent with a feedforward function of nNOS, which controls lactotrophs from excessive PRL release without affecting Ca^{2+} signals. Such a mode of regulation provides a steady nNOS and sGC activity, sustained messenger actions of NO, cGMP, and PKG, and renders operative other Ca^{2+}-influx controlled cellular functions. On the other hand, the spontaneous pacemaker activity is controlled by hypothalamic dopamine and somatostatin and pituitary endothelins, which abolish all voltage-gated Ca^{2+} influx-controlled cellular processes and thus serves as switch off for PRL secretion.

Acknowledgments

We are thankful to Drs. T. S. Kostic and L. Z. Krsmanovic for help in cyclic nucleotide measurements.

Received December 16, 2002. Accepted April 3, 2003.

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